The use of native and denatured recombinant coat protein forms for induction of good quality antisera for *Potato virus X* and *Potato leaf roll virus*

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Abstract

Polyclonal antibodies to recombinant (r) coat proteins (r CP) of *Potato virus X* (PVX) and *Potato leaf roll virus* (PLRV) were developed. The effectiveness of these antisera was determined by indirect ELISA (I-ELISA), immuno blotting, and double antibody sandwich (DAS) enzyme linked immunosorbent assay (ELISA), i.e. DAS-ELISA. The measured titers, with indirect-ELISA, expressed as reciprocal of the dilution end-points were 6000 for PLRV; whereas, PVX had a titer of 5000. The CP genes of PVX, and PLRV were amplified by reverse transcription-polymerase chain reaction (RT-PCR), cloned and expressed into pBAD-C terminal 6xHis Tag TOPO expression vector in *Escherichia coli*. For immunization, the CP fractions from bacterial lysates of each virus were purified, under native and denatured conditions, by nickel-nitrilotriacetic acid (Ni-NTA) batch chromatography and equally mixed. The fused proteins were 6.6 mg/20 ml for PVX and 9.0 mg/20 ml for PLRV bacterial culture. Antigenicity of the purified CP fraction was measured with Western blotting (WB) analysis. The r CP of each of PVX, and PLRV purified under naturing and denaturing conditions were injected into rabbits for antibody productions. The PVX- and PLRV-induced antisera reacted in I-ELISA, indirect plate trapped antigen enzyme-linked immunosorbent assay (IPTA-ELISA), and dot blotting immuno binding assay (DBIA). The data indicated that the produced r antisera were efficient and accurate in differentiation between negative and positive samples in DAS-ELISA upon testing leaves and sprouts of several potato varieties tests. Therefore, these r antisera are suitable for the certification program of potatoes due to their low cost, high specificity, feasibility, and their endless supply from r bacterial clones carrying the CP genes for these viruses.

Keywords: Potato; *Potato virus X*; *Potato leaf roll virus*; RT-PCR; recombinant coat protein antisera; immunodiagnosis; DAS-ELISA; IPTA-ELISA.

{Citation: Aly M. Abdel-Salam, Ahmed K. El-Attar, Ahmed M. Soliman. The use of native and denatured recombinant coat protein forms for induction of good quality of antisera for *potato virus X* and *potato leaf roll virus*. American Journal of Research Communication, 2013, 1(7): 70-86} www.usa-journals.com, ISSN: 2325-4076.

Introduction

PVX is the most widespread of potato viruses and often completely infects certain commercial stocks, causing yield reductions (Burrows and Zitter, 2005). PVX is non-enveloped, flexuous, filamentous, 470-800 nm or more long and 12-13 nm in diameter. PVX genome contains single stranded RNA capped, polyadenylated, and contains five open reading frames (ORFs) (Huisman *et al*., 1988). The coat protein gene is located near the 3'-end of the viral genome

PLRV is a major menace for the potato production all over the world (Ehrenfeld *et al*., 2004). PLRV is the type member of the genus *Polerovirus* of the Family *Luteoviridae* (Pringle, 1998). PLRV has a monopartite, single stranded RNA genome, transmitted by aphids in a circulative non-propagative manner and is mainly restricted to phloem tissues of infected plants (Mayo and Ziegler-Graff, 1996). PLRV forms 25 to 30 nm diameter isometric particles that encapsulate genomic RNA of about 5.9 kb that contains six large open reading frames (ORF)**.** Of these, the coat protein (CP) gene is located within the gene clusters at the 3'end and ORF3 encodes the 23 kDa PLRV coat protein

Serologic detection of plant viruses is crucial for counteracting disease epidemics. Serologic techniques such as ELISA performed either in liquid or solid phases can be employed effectively for accommodating large numbers of samples simultaneously with easiness, accuracy, and reasonable cost. Effectiveness of ELISA depends originally on the presence of good quality antisera with high titer, high immunogenecity, specificity, and low background.

Recombinant DNA technology may help circumvent purification problems encountered with several viruses (Ling *et al*., 2000; Abdel-Salam *et al*., 2004, 2005; Beuve *et al*., 2007; Fajardo *et al*., 2007; Khan *et al*., 2012). In additions, r antisera evade problems encountered with the presence of mixed infections usually lead to the presence of secondary

antibodies besides the target antibodies which complicates diagnosis as noticed with *Grapevine leafroll-associated virus* (GLRaV) infecting grapevine (Fajardo *et al*., 2007).

Certain problems, however, were raised upon using r antisera prepared by using denatured CPs instead of using intact CPs. For instance, some r antisera for certain closteroviruses, comoviruses, potyviruses, and tospoviruses, showed low specificity and/or high cross reactivity with other related viruses (Jacquet *et al*., 1998; Kalmar, and Eastwell, 1989, Bar-Joseph *et al*., 1997; Gera *et al*, 1999). In addition, many of the r antisera induced for denatured coat protein, though reactive in I-ELISA, did not react or reacted poorly in DAS-ELISA (Gera *et al*., 1999; Čerovska *et al*., 2003; Folwarczna *et al.,* 2008). DAS-ELISA is known for its high selectivity against cross reactive antisera (Agranovsky *et al*., 1994; Fajardo *et al*., 2007).

Recombinant antisera for Egyptian isolates of both PVX (Soliman *et al*., 2006) and PLRV (El-Attar *et al.,* 2010) were induced using denatured CP technology. These antisera were reactive in I-ELISA but not in DAS-ELISA. In the present study, we are describing a modified technique involving the use of a mixture of native and denature CP for each virus in the antiserum production to enhance the binding capacity of the produced antibodies with their corresponding antigens in DAS-ELISA.

Materials and Methods

Virus sources and field sample collections

The virus isolates in the present study were isolated from the Experimental Farms of the Agriculture Research Center, Ministry of Agriculture at Giza, Egypt. The PVX isolate was originally isolated and maintained by Soliman *et al.* (2006), while PLRV was isolated by El-Attar *et al*. (2010). Several potato varieties; viz. Annabella, Spunta, Nicola, Santyee, Marenka, and Mustang, Agro Plant, Lady Rosseta, Herems, and an unknown commercial potato variety were serologically tested for natural infection with PVX and PLRV using prepared r antisera (present study) and classical antisera for PVX and PLRV kindly supplied by P.E. Thomas Washington State Univ., Prosser, WA, USA.

Coat protein amplification and cloning

The CP genes of the Egyptian isolates of PVX and PLRV, in the present study, were amplified by RT-PCR using PVXCPVECORI: 5'-GATAGAATTCAGATGACTACACCAGCCAACACC-3' AND PVXCPCNCOI: 5'-TACGCGTCGGTTCCATGGACGTAGTTATGGTGG-3' (Soliman *et al*., 2006) and PLRVCPv: 5'-ATGAGTACGGTCGTGGTTARAGG-3' and PLRVCP*Nco*I: 5'-AAAACCATGGCTATYTGGGGTTYTGCARAGCTAC-3' (El-Attar *et al*., 2010), respectively. *Eco*RI enzyme restriction site (ERS) in forward primer (underlined) and *Nco*I ERS in reverse primer (underlined) for PVX CP and *Nco*I ERS in reverse primer for PLRV (underlined) were added for cloning purpose.

RT-PCR products were ligated directly into pBAD-TOPO® vector (pBAD-TOPO® TA Expression Kit) obtained from Invitrogen, Carlsbad, CA. The r plasmids were introduced into *E. coli* strain BL21 (DE3) as described by the manufacturer's instructions. DNAs were prepared from selected white colonies, digested with restriction enzymes and fractionated onto agarose gels using one Kb DNA Ladder marker (Promega). In other experiments the r plasmid-DNAs were amplified directly with PCR using the designed primers for each virus and tested on 1% agarose gel electrophoresis.

Expression and purification of the PVX and PLRV coat proteins.

Protein expressions were performed as previously described by Soliman *et al*. (2006) for PVX and El-Attar *et al*. (2010) for PLRV. Recombinant plasmids for PVX and PLRV were transformed into the *E. coli* BL21 cells, in the presence of 60 µg/ml ampicillin, and, incubated overnight at 37ºC with vigorous shaking to reach an OD_{600} of approximately 0.6. Different concentrations of L arabinose (0.00002%, 0.0002%, 0.002%, 0.02%, and 0.2%) were tested for the induction of CPs. Purification of the induced r-proteins was via both native and denaturing conditions in attempts to obtain antigenic viral external and internal CP epitopes for animal immunization. Purifications were performed using the Ni-NTA Fast Start Kit (Cat No. 30600,) by Qiagen as per manufacturer's instructions. Pellets from large scale cultures were thawed for 15 min on ice and re-suspended in lysis buffer (pH 8.0) containing lysozyme and benzonase. Suspensions were incubated on ice for 30 min with occasional swirling of the cell suspension, and then centrifuged 14,000 g for 30 min at 4°C.

Cell lysate supernatants, containing the soluble recombinant protein fractions, were transferred to new tubes. The remainder of the cell lysate was applied to a Fast Start column. The column was washed twice with four ml of wash buffer (pH 8.0) for native purification or pH 6.3 for denaturing purification. Bound 6XHis-tagged proteins were eluted with three-one ml aliquots of Elution buffer (pH 8.0, for native purification) or pH 6.3 (for denaturing purification). At each step, a five-micro liter sample was collected to monitor the purification process. Each sample was mixed with 2X SDS-PAGE buffers and stored at -20°C for SDS-PAGE. Elutes of r CPs, prepared under natured and denatured methods, were added altogether and dialyzed overnight at 4°C in 20 mM phosphate (Na_2HPO_4-NaH_2PO_4) buffer, pH 7.4. Protein concentration of the recombinant CPs were estimated by the spectrophotometer using an extinction coefficient = 1 (A_{280} = 1 for 1 mg/ml).

Western blotting analysis

Proteins separated by SDS-PAGE were electro-blotted onto nitro cellulose membranes (0.45 µm, Biobind-NC, Whatman, England) in a semidry system (Biometra, Germany) using 2.5 mA/cm^2 using a transfer buffer (25 mM Tris-base, 150 mM glycine and 10% methanol, pH 8.3). After blotting, the membranes were stained non-specifically with Ponceau S (Sigma-Aldrich, P3504, St Louis, Mo, USA) to mark the molecular weight bands of the protein marker (Sigma-Aldrich M.3913 St.Louis, Mo. USA). The membranes were then incubated for 1 h at 37°C in 5% non-fat dry milk and 1% bovine serum albumin, prepared in PBST, pH 8.0. For the detection of the r CP, the membranes were incubated overnight at 4°C with either antiserum of PVX or PLRV (P.E. Thomas), diluted 1/100 in PBS. The membranes were washed four times in PBST and incubated for 3 h at 37°C in alkaline phosphatase conjugated goat anti rabbit IgG (Sigma-Aldrich, A-3687, St Louis, Mo, USA) diluted 10^{-4} in ELISA conjugate buffer (PBST, pH 7.4, containing 0.2% BSA and 2% polyvinyl pyrrolidone, PVP). The membranes were washed six to eight times in PBST (phosphate buffer saline, PBS, containing 0.05% Tween 20) to remove non-bound secondary antibodies. The bands of interest were visualized by the Fast Red TR/Naphthol/naphthol AS-MX-phosphate complex (Sigma-Aldrich, A-3687, St Louis, Mo, USA).

Production and purification of antisera

For each virus understudy, immunization of rabbit involved injection with an equal mixture of fusion CPs prepared under native and denaturing purification conditions and suspended in 20 mM PBS and 0.1% Triton X100. The animal received five weekly consecutive intramuscular injections. Each injection was composed of a total of 600 µg fusion CP mixed (1:1, v/v) with Freund's complete adjuvant in the first-week injection. Subsequent injections used incomplete adjuvant instead. One week after the last injections, the rabbit was bled. The serum fraction of the blood was separated through centrifugation at 1300 rpm for 15 min at 4°C then stored at -20^{o}C. The IgG fraction was precipitated from the crude antisera by caprylic acid and ammonium sulphate according to the method of Mckinney and Parkinson (1987). The concentration of IgG of each virus was measured at 280 nm (OD of 1.4 = 1 mg/ml) with 0.5 X PBS. The measured IgGs were frozen at –20^{o}C.

Immunoassay tests

Indirect (I)-ELIA was used according Converse and Martin (1990) for measuring titer of the PVX- and PLRV-recombinant antisera.

Indirect plate-trapped antigen (IPTA)-ELISA was used to detect potato infected plants using r CP antisera according to Folwarczna *et al*. (2008). Microtitre plates were coated with antigens diluted 1/10 in standard carbonate coating buffer (pH 9.6) and incubated overnight at 4 °C. The ELISA plates were washed four times with PBST and incubated for 2 h at 37 °C with 4 µg/ml IgG diluted in conjugate buffer. The plates were washed and incubated with goat anti-rabbit alkaline phosphatase conjugate (Sigma A-8025) diluted at 10^{-4} in conjugate buffer. Plates were given 6 times washing prior to substrate addition.

Dot blotting immunosorbent assay, DBIA, was used, as described by Abdel-Salam *et al*. (1997), to confirm detection of the expressed PVX-CP and PLRV-CP antigens and to detect both virus isolates in infected potato tissues.

DAS-ELISA was used to test the presence of PVX and PLRV in leaves and sprouts of several potato varieties in two separate experiments. Basically the protocol described by Clark and Adams (1977) was followed with minor modifications where a blocking step (1 hr/ 37^{o}C) using a blocking solution composed of 5% non-fat dry milk (w/v) and 1% (w/v) BSA was added after coating the plates with different dilutions of purified γ-globulin IgG;

viz, 4 µg/ml, 2 µg /ml, and 1 µg /ml, prepared in coating buffer (pH 9.6). The antigens were detected by incubation (3 hr at 37°C or overnight at 4°C) with different dilutions; viz. 1/250, 1/500, 1/1000, up to /10,000) of r-PVX- or r-PLRV-IgG labeled with AP.

Results and Discussion

Amplification and cloning verification of the CP genes of PVX and PLRV

RT-PCR amplified the full CP genes of PVX (~750 bp) and PLRV (~ 650 bp). DNA preparations of charged plasmids retrieved from transformed bacterial colonies and digested with restriction enzymes confirmed the transformation of CPs of PVX and PLRV as previously described by Soliman *et al*. (2006) and El-Attar *et al.* (2010), results not shown.

Generation of recombinant PVX- and PLRV-CP antisera

The CP gene for each of PVX and PLRV in the r plasmids was expressed in *E. coli* with L arabinose and purified under native and denaturing conditions. The non-induced culture produced a lower amount of protein than induced ones as expected (Fig. 1,A & B) The total yield of CP fusion protein fractions, from both methods, was 6.6 mg/20 ml bacterial lysate for PVX and 9.0 mg /20 ml mg bacterial lysate for PLRV culture.

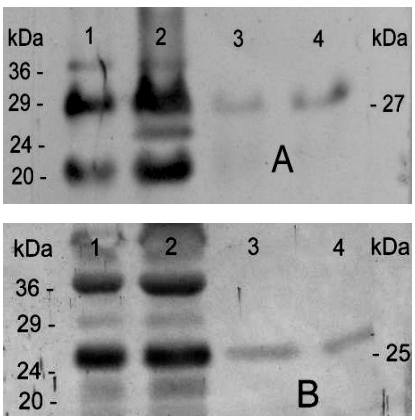

Fig. (1): Western blot analysis of parallel runs of SDS-PAGE recombinant CPs expressed in *E. Coli* strain BL21. Lane 1, un-induced culture; Lane 2, 0.0002 % L arabinose induced culture; lanes 3 and 4, eluted fractions 1, 2, respectively of purified PVX-CP (A) and PLRV-CP (B) after Ni-NTA chromatography. The 27 kDa in (A) represents the 25 kDa of PVX-CP plus 2kDa of tagged fragment from the vector. The 25 kDa in (B) represents the 23 kDa of PLRV-CP plus 2 kDa of tagged fragment from the vector.

The fusion CPs of PVX and PLRV reacted with their corresponding antisera in Western blotting analysis. The purified fusion PVX-CP measured 27 kDa (Fig. 1-A). The 27 kDa represents the 25 kDa of PVX-CP (Soliman *et al.* 2006) plus 2 kDa of tagged fragment from the vector. The 25 kDa band of PLRV-CP in Fig.1–B represents the 23 kDa of PLRV-CP (El_Attar *et al*., 2010) attached to the 2 kDa of tagged fragment from the vector.

Evaluation of the recombinant PVX- and PLRV-CP antisera for virus detection

The efficacy of r PVX- and PLRV-CP antisera in detecting their corresponding antigens were evaluated by measuring their titers using: I-ELISA, IPTA-ELISA, and DBIA tests.

The measured titers, with I-ELISA, expressed as reciprocal of the dilution endpoints was 5000 for PVX and 6000 for PLRV. This method was previously shown to be useful in detection of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* with their r antisera (Lee and Chang, 2008).

IPTA-ELISA was successful in detection of PVX and PLRV in leaves and tuber sprouts of several potatoes varieties (Figs.2 &3), respectively. Recombinant fusion CPs of PVX and PLRV were used as positive controls in IPTA-ELISA to test their antigenicity against immunogenicity of their corresponding r antisera. Additionally CPs of PVX and PLRV were tested at known concentrations in Figs.2 and 3 to allow rough estimation of virus concentrations in other tested potato varieties at O.D. $_{405\ nm.}$ Such estimation of virus concentration against optical density is well known in quantitative ELISA and used to measure varietal resistance against corresponding antigens. IPTA-ELISA was used by several investigators for evaluating r antisera of *Potato mop top virus* in potato (Cerovska *et al*., 2003) and PVY in potato (Folwarczna *et al.*, 2008; Abdel-Salam *et al*., 2013).

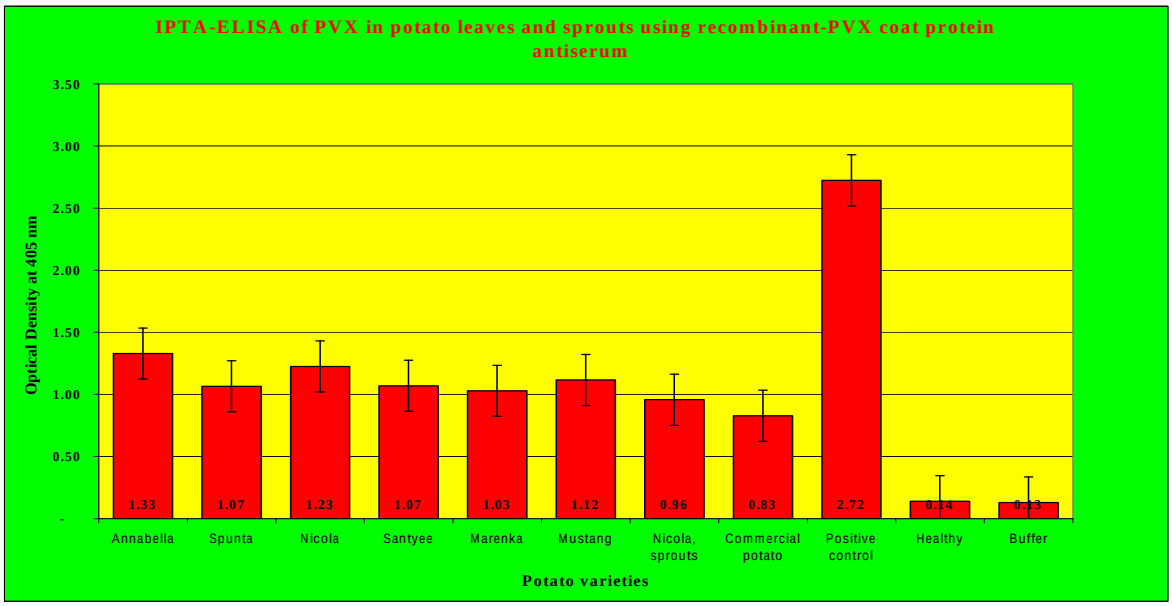

Fig. (2): IPTA-ELISA of PVX in potato leaves and sprouts using r PVX coat protein antiserum. Each reading represents an average of ten replicates. IgG dilution=1/250, GAR-AP dilution= 10^{-4}. A positive result was taken as an absorbance (at 405 nm) of three times the mean of the corresponding healthy control. Positive control represents PVX fusion coat protein diluted 10^{-1} equal to 66 µg/ml. R^2 =0.084

DBIA tests were used successfully for measuring the antigenicity of the r CPs of PVX and PLRV with their r antisera (Fig.4) as well as with antisera for PVX and PLRV prepared with the classical methods (Fig.5). The immunogenicity of the r antisera for PVX and PLRV were also examined for detecting these viruses in commercial potato plants the field (Fig.6). Similar results showed the high antigenicity of r CP of *Potato virus Y* and immunogenicity of its r antiserum upon evaluation with DBIA (Abdel-Salam *et al*., 2013).

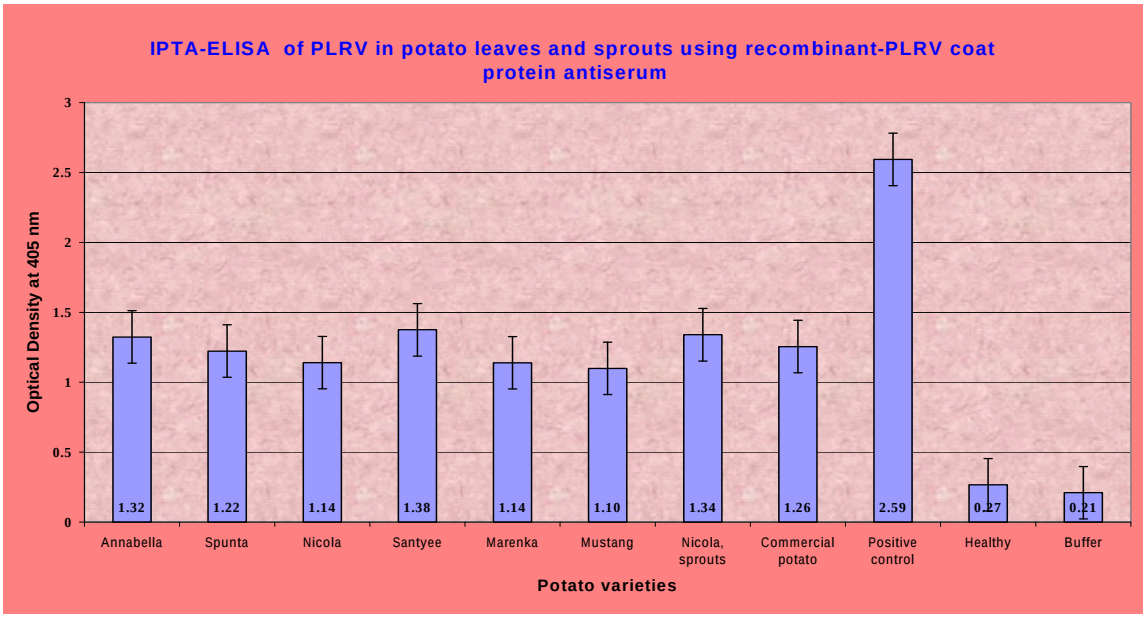

Fig. (3): IPTA-ELISA of PLRV in potato leaves and sprouts using recombinant-PLRV coat protein antiserum. Each reading represents an average of ten replicates. IgG dilution=1/250, GAR-AP dilution= 10^{-4}. A positive result was taken as an absorbance (at 405 nm) of three times the mean of the corresponding healthy control. Positive control represents PLRV fusion coat protein diluted 10^{-1} equal to 90 µg/ml. R^2 = 0.0605.

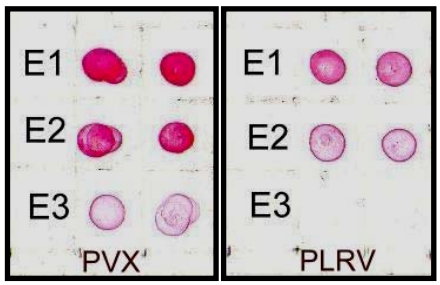

Fig. (4): BIA showing the serologic reactions between r fusion coat proteins, CP, (Elutions, E1, E2, and E3, of PVX and PLRV with their corresponding r antisera. 1 µl of protein suspension was applied /spot. Recombinant IgGs were used at 1/250, dilution and GAR-AP at 10^{-4}. Nitrocellulose membranes (NCM) were stained with Naphthol/Fast Red complex

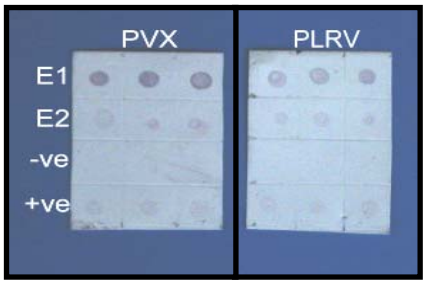

Fig. (5): DBIA showing the efficacy of r-coat proteins of PVX and PLRV in reaction with their corresponding authentic polyclonal antisera (P.E.Thomas, Washington Stat University, Prosser, USA. E1 and E2 are purified recombinant proteins; -ve, sap of healthy potato; +ve, sap from infected potato. 1 µl of protein suspension was applied /spot. Antisera were used at 10^{-3} dilutions and GAR-AP at 10^{-4}. NCM were stained with NBT/BCIP complex.

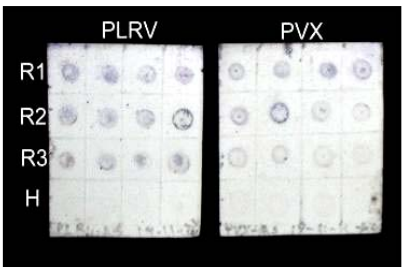

Fig. (6): DBIA showing the efficacy of the induced r antisera for PLRV and PVX in detection of these viruses in potato leaves (row, R1, R2, and R3) picked up randomly from commercial potato fields. H, represents healthy potato samples. Each square on the NCM represents a separate sample. 1 µl of tested sap diluted 10^{-1} with coating buffer was applied /square. Antisera were used at 10^{-3} and GAR-AP at 10-^{4}. NCM were stained with NBT/BCIP complex

Evaluations of the r antisera of PVX and PLRV were performed using DAS-ELISA. Several concentrations of IgG and IgG-AP conjugate were tested for DAS-ELISA optimization. Dilutions 1/250 and 1/500 were the optimal dilutions for the PVX-IgG and IgG-AP conjugate, respectively. Whereas, optimal dilutions for PLRV-IgG and IgG-AP conjugate were at 1/250 dilution.

Results in Tables (1 and 2) indicated the efficacy of the recombinant antisera of both viruses in detection of their antigens in leaves and sprouts of several potato varieties. No background was detected in healthy potato samples; indicating the superiority of the r antisera of both viruses when tested with DAS-ELISA.

Table (1): DAS-ELISA of potato leaves and sprouts against the presence of PVX and PLRV using their recombinant antisera

Optical Density (O.D) at 405 nm*								
Tested viruses	Tested samples**							
	Potato varieties				Controls			
	Leaves			Sprouts	Infected leaves (+ve)	CP	Healthy leaves (-ve)	Buffer
	Annabella	Sponta	Nicola	Unknown variety				
PLRV	0.520	0.256	0.471	1.965	0.661	0.928	0.010	0.009
PVX	0.522	0.200	0.615`	1.021	0.548	0.753	0.052	0.038

* A positive result was taken as an absorbance (at 405 nm) of 3 times the mean of the corresponding healthy control. **Data represent the mean of five replicates. LSD (least significant difference) measured at p = 0.5 (using the ANOVA: Single Factor Program of Microsoft Office Excel 2010) was 0.336 for PLRV and 0.22 for PVX. CP (50 µg/ml) for each virus was used as a control antigen to check the validity of the recombinant antisera.

Table (2): DAS-ELISA of potato sprouts against the presence of PVX and PLRV using their recombinant antisera

Optical Density (O.D) at 405 nm*						
Tested viruses	Tested samples**					
	Potato varieties			Controls		
	Agro Plant	Lady Rosetta	Hermes	Infected tubers (+ve)	Healthy tubers (-ve)	Buffer
PLRV	1.034	1.151	1.426	1.185	0.093	0.093
PVX	0.452	0.527	0.717	1.112	0.087	0.097

* A positive result was taken as an absorbance (at 405 nm) of 3 times the mean of the corresponding healthy control. **Data represent the mean of five replicates. LSD (least significant difference) measured at $p = 0.5$ (using the ANOVA: Single Factor Program of Microsoft Office Excel 2010) was 0.175 for PLRV and 0.101 for PVX.

In the present study the r antisera for PVX and PLRV were induced through immunizing the animal with equal concentrations of virus CP prepared under denatured and native conditions. This would expose both epitopes (outer epitopes) and cryptopes (hidden epitopes) to the animal immune system and therefore enhance immunogeneictiy.

The conformational status of the viral-structural protein defines its relation with its induced antibodies (Mernaugh *et al*., 1993). For instance viral-denatured protein of *Faba bean necrotic yellows virus* was only expressed as discontinuous epitopes (cyptopes) and reacted poorly with its corresponding antiserum prepared for native coat protein, i.e. whole virus, (Abdel-Salam *et al*., 2004). As for other viruses as *Banana bunch top virus* enjoying the presence of continuous epitopes, its induced antiserum, prepared from denatured protein, did not suffer from poor serologic reactivity (Abdel-Salam *et al*. 2004).

Previous reports indicated that some r antisera induced only for denatured proteins in potyviruses (Jordan and Hammond, 1991; Folwarczna *et al*., 2008), or comoviruses (Gabriel *et al*., 1989), potato mop top furovirus (Čerovska *at al*., 2003) suffered from high cross reactivity on one hand and or poor reactivity on the other hand due to surface-protein alterations. High cross reactivity, and hence low specificity, of the r antiserum would allow its cross reaction with other related viruses in I-ELISA but not DAS-ELISA; since the latter test is highly specific for its direct binding of antibodies to their corresponding antigens. This perhaps explains, in turns, the poor or negative reactions of some r antisera, prepared from denatured structural proteins, in DAS-ELSA (Cerovska *et al.,* 2003; Soliman *et al*., 2006; Folwarczna *et al*., 2008).

Other investigators working with other viruses included, therefore, native and denatured antigens (Abdel-Salam *et al*., 2013) or used boosting injections of native antigens during immunization with denatured antigens to enhance antigen-antiboy binding (Jordan and Hammond, 1991; Jacquet *et al.,* 1998; Bar-Joseph *et al*., 1997; Gera *et al*., 1999).

Conclusion

From the above narrative discussion, it would be advisable upon preparing r antisera to include both forms of CP purified under native and denatured conditions side by side to alleviate the unawareness of the conformational status of the viral-structural protein understudy. Further, to increase immunogenicity of the induced antisera as was done in the present study. The induced r antisera for both PVX and PLRV were highly immunogenic, had high titer and showed good efficacy in DAS-ELISA upon reaction with their corresponding antigens in leaves and sprouts of several potato varieties. This should give

invaluable importance in using these two antisera in quarantine stations and for indexing for virus-free potatoes.

Acknowledgements

The authors would like to offer their gratitude to Dr. P.E. Thomas, Washington State University, Prosser, USA, for donating classical polyclonal antisera for PVX and PLRV.

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